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From the Russian for
Dr. John F. Bell

Trudy Rostovskogo na Donu
gosudarstvennogo n.-i.
protivochnogo in-ta. IV,
51-63, 1945.

Zhidkaya zheltchnaya sreda dlya vyrashchivaniya
B. tularense

Liquid egg yolk culture medium for
Bacterium tularense

by

M. S. Drozhevskina

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The culturing of Bacterium tularense in artificial media presents considerable difficulties, inasmuch as this microorganism requires a medium containing special nutritive components. Bacterium tularense fails to proliferate unless the medium includes certain specific albumins (in a special state of degradation), plus carbohydrates, cysteine, and lecythin, all of them combined in certain strictly defined proportions. Bacterium tularense is characterized among others by a sharply reduced synthesizing ability, a fact which does not allow it to grow in ordinary culture media.

Bacterium tularense was cultured for the first time on an artificial medium by McCoy, who used for this purpose the type of coagulated egg yolk medium usually employed for Mycobacterium tuberculosis cultures. The McCoy and Chapin medium is still preferred for growing Pasteurella tularensis, but it has some serious shortcomings. For example, this particular microorganism grows in it very slowly; on having been isolated from the animal source and transferred to the medium, it usually requires from 2 to 3, sometimes 4 - 12 days,

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before starting to proliferate (Miller, Khatenever, Pokrovskaya, Sinay, Levchenko, etc.). In many cases it cannot be grown from dead animal material in the coagulated egg yolk medium and cultures can be obtained only by repeated animal passages. Furthermore, the McCoy medium requires large quantities of egg yolk and a preparation method complicated by the fact that the temperature of the coagulant has to be closely watched, that the medium has to be checked for water of condensation, etc.

All of which has led many researchers to investigate other culture media in the hope that one of them would prove simpler to prepare and more practical in use. Many media were proposed including Francis' medium (cysteine agar with sugar and blood), ascitic fluid agar with glucose, sugar blood agar, cysteine sugar with fish hydrolyzate. Khendlesson's 5% glycerin and liver agar; a medium usually employed for culturing the brucellosis agent, was proposed by the workers of the Rostov Microbiological Institute for the propagation of laboratory strains of *Bact. tularensis*. They also studied the possibility of developing vitaminized culture media, a carrot medium, in particular. Vereninova investigated various media with a high vitamin content and found that Bailey's liver agar was best for *Bact. tularensis*. She found that the growth of the latter is considerably enhanced by a supplement of glucose and cysteine. However, this medium is less appropriate for culturing *Bact. tularensis* strains newly isolated from animal sources. Hence, she feels that while *Bact. tularensis* can be preserved and propagated in this medium, the latter can not serve as a primary isolation medium. Korobkova has recently proposed a new type of propagation medium. This medium contains 1/5th yeast autolyzate instead of the fish hydrolyzate. Korobkova has been able to modify this medium by replacing the agar with starch. It should be added that all the media mentioned in the preceding are of the solid type.

It is still being debated whether *Bact. tularensis* can be cultured in liquid media. Many authors (in particular Danna and Bond) feel that it cannot. Karpov, Miller, Dorofeyev and others, state that it produces a floccose growth in bouillon containing a supplement of glucose and serum. Vereninova studied the growth of *Bact. tularensis* in a liquid medium, namely in a cysteine bouillon containing both glucose and serum. With inoculation doses of 100 million, 250 million and 500 million bacterial bodies she saw no proliferation, but on

using much larger doses (1 - 2 billion bacterial bodies) she noted within 2 - 3 days that the material in the test tubes was becoming cloudy, that there was hydrogen sulfide formation, and the fact that the pH of the culture medium rose from 7.4 to 7.7.

Further experiments revealed that on being transferred to this type of bouillon, *Bact. tularensis* does not proliferate, but begins to die off after the 4th day.

However, after having supplemented this culture medium with 0.1% of agar, she obtained excellent results even with inoculation as low as 1,000 bact. bodies per cubic centimeter of the medium. This favorable effect of agar on the growth of *Bact. tularensis* is explained by a statement found in Smorodintsev's work. The latter writes, basing his statement on findings regarding the spores of the Siberian ulcer, which proliferate in this type of bouillon when the latter contains some agar, but fail to proliferate when there is no agar, that the physical state of the culture medium determines how the cultured bacteria will utilize the nutrients offered by the medium. A 0.5% addition of "protective-colloid" agar-agar has a favorable, growth-stimulating effect on many types of microorganisms. Apparently, *Pasteurella tularensis* is one of them. Smorodintsev is of the opinion that a good culture medium can be prepared only by considering both the nutritive characteristics required, and the physical state of the medium.

In her last work on the microbiology of *Bact. tularensis*, Vereninova writes that this microorganism can grow in an artificial culture medium only if the latter contains the growth-stimulating substances that are found in the egg yolk, plus vitamin B (complex), as occurring in yeast. She adds that another component indispensable for the growth of *Bact. tularensis* is a protective colloid.

Sinay published his work in 1935. He was first to use a liquid culture medium composed of 60% egg yolk and 40% physiological saline solution. Though prepared according to McCoy and Chepin's basic formula, this medium was not of the coagulated type. Sinay inoculated this medium with material from the primary sites of infection, i.e. with bubonic punctate and blood from human sources. At the same time, he used the same material to infect rabbits.

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His study was conducted on a total of 8 rabbits. All of them died presenting typical anatomico-pathological symptoms. The cultures in the liquid medium were submitted to examination after 26 days. All attempts to subculture the bacterium were negative, but 6 of the animals which had been infected by means of the inoculated culture medium died and yielded pure Bact. tularensis cultures. Hence, the author concludes that the tularemia agent retains its pathogenic properties in the liquid egg yolk medium but not its faculty to proliferate, and therefore, that the liquid medium should be employed as a preserving medium whenever the disease cannot be immediately transmitted to animals.

Preparing the medium. Selection of optimum egg yolk concentration.

The liquid medium is very simple to prepare. Like McCoy's medium, it is composed of chicken egg yolk and physiological solution. If prepared under sterile conditions and in sterile equipment, it requires no additional sterilization, and the percentage of overgrowth is negligible, seldom exceeding 3 - 4%. The bottled medium is placed in the thermostat for two days to make sure it is sterile. The medium was prepared by the author in a series of batches containing different percentages of egg yolk, thus, 5%, 10%, 20%, 30%, and 40%. Certain parts of the 5, 10 and 30% batches were put through a Seitz filter and distributed into test tubes. The test tubes with the filtered samples, as well as others containing the unfiltered medium are placed after having been inoculated with varying doses of three Bact. tularensis strains (Nos. 9 and 11, two virulent strains, and No. 10, an avirulent strain) in the thermostat, where they were left for 10 days at a temperature of 37°. After having been removed from the thermostat, they were left at room temperature. Their contents were daily sampled and the material was made into smear preparations and used for starting subcultures in McCoy's medium.

The results of these tests are summarized in Table 1.

Table 1 shows that in the 10% medium, regardless of the inoculation dose, there is active bacterial growth already after 24 hours. A single loop of this medium, if transferred to McCoy's medium, produces on it a solid layer of bacterial growth. It is worth mentioning that the 24-hour growth of strain No. 11 in the 10% medium was better than in the 20% and 30% media.

In the 5% medium, bacterial proliferation could be observed during the early days only with massive inoculation doses. Around the 10th day, all the 5% test tube cultures showed bacterial growth, but not as active as that presented by test tubes containing other egg yolk concentrations.

Proliferation in those test tubes which contained samples of the filtered medium was much poorer than in test tubes containing the non-filtered medium. Apparently, the filter retains certain substances which are essential if *Bact. tularensis* is to grow.

On the strength of her findings, the author has come to the conclusion that the 10% concentration is best: regardless of the size of the inoculation dose it produces, even in 24 hours, a higher number of cultures than the other concentrations.

When microscopically examined, the 10% medium appears almost homogeneous; and clearly brings out the *Bact. tularensis* even in Gram-stained smear preparations. The 30% and 40% media, on the other hand, are of a coarse texture so that in preparations stained by Gram's method, the bacterium is not readily distinguished from the surrounding granulations.

The 10% medium is superior, because cultures can be isolated in it within a very short time and because it is more economical, inasmuch as it requires small amounts of egg yolk. Actually, one egg yolk yields enough culture medium to supply 30 to 40 test tubes.

Determining the minimum *Pasteurella tularensis*
inoculation dose in the liquid medium.

The superiority of the liquid over the coagulated egg yolk medium has been experimentally demonstrated. The first two experiments were conducted using three different strains of *Bact. tularensis*. Then the same experiments were repeated using 9 other strains obtained from various sources. Procedure: A series of test tubes, each containing some 5 cc of the 10% culture medium, were seeded with predetermined bacterial doses according to *e.-coli* standard. For the first 10 days, the seeded test tubes were left in the dark, then kept at room temperature. Their contents were daily sampled and the sample material was

Table 1.
Selecting the optimum egg yolk concentration in the medium.

| % of egg yolk in medium | Str. No. | Inoculation dose | | | | | | | | | | 5-day cultures | | | | |
|----------------------------|-------------|------------------|-----------------|----------------|---------------|-------------|-----------------|----------------|---------------|-------------|-----------------|----------------|---------------|-------------|-----------------|----------------|
| | | 1-day cultures | | | | | 100,000 | | | | | 1 mill | | | | |
| | | 1 mill. b.b. | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 100 b.b. | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 100 b.b. | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 100 b.b. | 100,000 b.b. | 10,000 b.b. |
| 5 | 10 | v | v | v | - | - | - | - | - | - | - | - | - | - | v | - |
| | 11 | v | v | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 9 | v | v | v | - | - | - | - | - | - | - | - | - | - | v | - |
| 10 | 10 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 11 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 9 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| 20 | 10 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 11 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 9 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| 30 | 10 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 11 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 9 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| 40 | 10 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 11 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| | 9 | g | g | g | g | g | g | g | g | g | g | g | g | g | g | g |
| Controls, McCoy's m. | 10 | v | - | - | - | - | - | - | - | - | - | - | - | - | g | - |
| | 11 | - | - | - | - | - | - | - | - | - | - | - | - | - | g | - |
| | 9 | v | - | - | - | - | - | - | - | - | - | - | - | - | g | - |
| Filtered 20 | 10 | v | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 11 | v | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 9 | v | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Symbols: v = weak growth; g = satisfactory growth; s = good growth; vg = very good growth; - = no growth.

(Table continued on following page)

Table 1 continued

| % of egg yolk in medium | Strain number | 10-day cultures | | | | | |
|----------------------------|------------------|------------------|-----------------|----------------|---------------|-------------|----|
| | | Inoculation dose | | | | | |
| | | 1 mill. b.b. | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 100 b.b. | |
| 5 | 10 | s | w | w | w | w | w |
| | 11 | s | w | w | w | w | w |
| | 9 | s | s | w | w | w | w |
| 10 | 10 | vg | vg | vg | vg | vg | vg |
| | 11 | vg | vg | vg | vg | vg | vg |
| | 9 | vg | vg | vg | vg | vg | vg |
| 20 | 10 | vg | vg | vg | vg | vg | vg |
| | 11 | vg | vg | vg | vg | vg | vg |
| | 9 | vg | vg | vg | vg | vg | vg |
| 30 | 10 | vg | vg | vg | vg | vg | vg |
| | 11 | vg | vg | vg | vg | vg | vg |
| | 9 | vg | vg | vg | vg | vg | vg |
| 40 | 10 | vg | vg | vg | vg | vg | vg |
| | 11 | vg | vg | vg | vg | vg | vg |
| | 9 | vg | vg | vg | vg | vg | vg |
| Controls, McCoy's m. | 10 | s | s | s | w | - | - |
| | 11 | s | s | s | w | - | - |
| | 9 | s | s | s | w | - | - |
| Filtered 20 | 10 | w | w | - | - | - | - |
| | 11 | w | w | w | - | - | - |
| | 9 | w | - | - | - | - | - |

Symbols: w = weak growth; s = satisfactory growth; g = good growth
vg = very good growth; - = no growth.

used for smear preparations. In a parallel control test, test tubes with McCoy's coagulated egg yolk medium were inoculated with the same doses of the bacterium.

The experimental results in question are given in Table 2. They show that Bact. tularensis grows better in the liquid medium than in McCoy's medium: in the latter, the first signs of growth appear only after 2 - 3 days, while in the liquid medium the cultures are so well developed, in general, within 24 hours, that even one loop of the material transferred to McCoy's medium produces a dense growth on the latter. In these tests, the minimum inoculation dose which was established for most (8 out of 9) of the strains tested was approximately 5 bacterial bodies (by the e.-coli standard), whereas on McCoy's medium, the minimum dose was 500 bacterial bodies for 5 of the strains and 1,000 b.b. for the remaining.

Hence, these experimental results warrant the statement that the liquid medium which was used by the author requires an inoculation dose 100 times lower than the McCoy's medium. Also, that it produces bacterial proliferation within 24 hours, while McCoy's medium produces it only on the 2nd or 3rd day.

Years of experience have brought Korobkova to the conclusion that "the better the culture medium, the smaller the inoculation dose capable of producing a culture of a given microorganism in this medium."

Vereninova's experimental findings prove that in semi-liquid cysteine agar the minimum inoculation dose is 1,000 bacterial bodies, and in cysteine bouillon supplemented with serum and glucose, 1 billion b.b. According to various writers, including the author herself, the minimum inoculation dose on McCoy's medium is 500 b.b.

The liquid egg yolk medium proposed by the author produces colonies from inoculation doses of 50 or even 5 b.b. Which proves that it is superior to all the other media now being used.

Viability of Bact. tularensis remaining in the liquid medium for long periods of time without reseedling

The question whether Bact. tularensis survives in the liquid medium when kept in the latter for protracted periods of time has

received considerable attention. For example, according to Khatenover and Levchenko, in sealed test tubes on coagulated egg yolk medium, it survives at room temperature for 2 to 2-1/2 months without reseeding. O'Hara states that cultures kept on a solid medium at room temperature survive for two weeks without difficulty, but that after 3 - 4 weeks under these conditions and without reseeding, they no longer can be subcultured.

The viability of *Pasteurella tularensis* was studied on three strains cultured in the liquid medium. In this test a total of 110 test tubes had been seeded with different inoculation doses. These test tubes were kept in closets, at a summertime room temperature (24 - 30°C). The viability of the cultures was tested every two weeks by transferring some material from each of the test tubes to McCoy's medium.

Table 3 demonstrates that the bacteria started to die off gradually after remaining for two months in the liquid medium, although after 5 months, the test tube strains were still able to produce colonies from minimum inoculation doses. Conversely, the control bacteria grown in McCoy's medium, under identical conditions, all died within a month. Hence, it has been experimentally demonstrated that *Pasteurella tularensis* survives for a long time in the liquid medium, even when survival conditions are unusually poor.

The 10% liquid egg yolk culture medium: Growth characteristics and morphology of *Pasteurella tularensis* in the medium.

From the practical point of view, it is extremely important to know that the liquid culture medium in question can be prepared in advance in large quantities and then supplied, as the need arises, to epidemiological teams working in the field and to outlying epidemiological laboratories.

The preliminary findings of some experiments set up to determine how long the liquid culture medium can be used after it has been prepared indicate that its keeping properties are quite satisfactory. For example, bacterial growth in this medium after the latter had been kept in test tubes under the usual storage conditions (in closets) for 2, 3 and even 4 months, and then inoculated with *Bact. tularensis*, was as active as in the freshly prepared medium.

The 10% egg yolk medium, when bottled is pale yellow, opaque and slightly opalescent in the incident light. After the freshly prepared medium has stood for one or two days, a thick, viscous deposit settles at the bottom of the test tube. Apparently, however, this is of no importance as far as the *Bact. tularensis* - growing characteristics of the medium are concerned, since bacterial growth is as good in test tubes containing the deposit as in those where there is none.

Pasteurella tularensis produces no changes in the medium as it proliferates in it. It proliferates spreading in a diffuse manner, and forming no surface pellicle and no bottom deposit.

Examined in smears prepared with the liquid medium, *Pasteurella tularensis* shows an elongated form and appears larger in size than when cultured in McCoy's medium.

On carefully inspecting the microorganism in question in liquid medium smear preparations, the author observed (especially during the first 15 to 24 hours after seeding) a large number of bacteria arranged in pairs and seemingly linked together by long, delicate filaments. These thread-like shadows connecting separate bacteria were already mentioned by Khatenever and O'Hara. O'Hara is of the opinion that these filaments are a special, characteristic feature of *Pasteurella tularensis*. When these threads are present, it means that the process of bacterial proliferation is very active. Sometimes, one finds the bacteria arranged in fours and fives, forming little chains. By and by, as the bacterial population continues to proliferate in the medium, the bacteria begin to form little groups. It is mentioned by Khatenever, Miller and others, that in the liquid medium *Pasteurella tularensis* shows various forms (particularly vibrioid, elongated and twisted forms), and also that the changed microbial forms stain unevenly, showing inside the body one or more deeply stained granules. However, when cultures containing such forms are transferred to the coagulated egg yolk medium, they produce bacteria which are typical from the bacteriological point of view. Altered forms of the type mentioned have been observed in test tubes which had been allowed to stand for a long time. Examination of smears prepared with material from these test tubes showed that while the first forms cultured in them were normal, by the 24th or 25th day the cultures were almost entirely composed of the altered forms. Later, however, the number of the atypical forms decreased so that after another 5 or 6 days the

bacteria appearing in the smears were all typical.

The capsule of *Pasteurella tularensis* is mentioned by Khatenever, Bereninova, Karpov, and others. According to Siney, the capsule is more readily observed in smears prepared with serum suspensions than with physiological solution suspensions. Stained in liquid egg yolk culture smears, the capsule appears clearly against the pale pink background provided by the medium. Furthermore, the capsule shows better in liquid egg yolk smears prepared with cultures grown from organ fragments.

Animal experiments

After having ascertained that *Pasteurella tularensis* can be cultured in the liquid egg yolk medium, the author performed a series of experiments on animals. The purpose of these studies was to determine whether time could be gained in establishing the bacteriological diagnosis of tularemia by seeding the liquid medium with material from the organs of dead or diseased animals, and omitting animal passages. She realized, at the same time, that the rodents reaching the laboratory table would be apt to present different stages of the disease.

Pasteurella tularensis cultures isolated from animal sources by seeding the liquid culture medium with organ fragments.

The animal material in these studies consisted of laboratory animals which had died of tularemia after having been experimentally infected with the disease. The organs (spleen, glands, liver, lungs) of the animals were removed, treated with alcohol and singed. Then two more or less equal pieces were cut from the center of each organ and used, one, to inoculate the liquid egg yolk medium, the other to inoculate McCoy's medium.

The animals tested by this method numbered ten (8 white mice, 2 guinea pigs). In the liquid medium, the first signs of bacterial proliferation were generally observed within 24 hours, in McCoy's medium only on the 2nd and 3rd day. Table 4 presents the data on white mouse No. 100 and guinea pig No. 97, two cases which are cited as examples.

Table 2
Determining the minimum inoculation dose of *Pasteurella tularensis* in liquid egg yolk medium.

| Strain number | Culture medium | Twenty-four hour cultures | | | | | | | | | | Five-day cultures | | | | | | | | | |
|---------------|----------------------------------|---------------------------|-------------|------------|----------|----------|--------------|-------------|------------|----------|----------|-------------------|-------------|------------|----------|----------|--------------|-------------|------------|----------|----------|
| | | 100,000 b.b. | | | | | 10,000 b.b. | | | | | 100,000 b.b. | | | | | 10,000 b.b. | | | | |
| | | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 500 b.b. | 100 b.b. | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 500 b.b. | 100 b.b. | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 500 b.b. | 100 b.b. | 100,000 b.b. | 10,000 b.b. | 1,000 b.b. | 500 b.b. | 100 b.b. |
| 9 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 10 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 11 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 12 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 13 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 14 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 15 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 16 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| 17 | Liquid egg yolk m. McCoy's m. | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |

Symbols: + = First signs of growth
- = no growth

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Table 3. Viability of the tularemia agent kept for long periods of time in 10% liquid egg medium without re-inoculation.

| Strain number | Inoculation dose | Kept for: | | | | | | | | | |
|---------------|------------------|-----------|------|-------|-------|--------|--------|--------|--------|---|---|
| | | 2 wks | 1 mo | 6 wks | 2 mos | 10 wks | 3 mos. | 14 wks | 4 mos. | | |
| 9 | 1 mill b.b. | + | + | + | - | - | - | - | - | - | - |
| | 100,000 b.b. | ++ | ++ | - | - | - | - | - | - | - | - |
| | 10,000 b.b. | + | + | + | + | - | + | - | - | - | - |
| | 1,000 b.b. | ++ | ++ | + | + | + | + | + | + | + | + |
| | 100 b.b. | + | + | + | + | + | + | + | + | + | + |
| | 1 mill. b.b. | ++ | ++ | - | - | - | - | - | - | - | - |
| 10 | 100,000 b.b. | + | + | + | + | - | - | - | - | - | - |
| | 10,000 b.b. | ++ | ++ | - | - | - | - | - | - | - | - |
| | 1,000 b.b. | + | + | + | + | + | + | + | + | + | + |
| | 100 b.b. | ++ | ++ | + | + | + | + | + | + | + | + |
| | 1 mill. b.b. | + | + | + | + | + | + | + | + | + | + |
| | 100,000 b.b. | ++ | ++ | - | - | - | - | - | - | - | - |
| 11 | 10,000 b.b. | + | + | + | + | + | + | + | + | + | + |
| | 1,000 b.b. | ++ | ++ | + | + | + | + | + | + | + | + |
| | 100 b.b. | + | + | + | + | + | + | + | + | + | + |
| | 1 mill. b.b. | + | + | + | + | + | + | + | + | + | + |
| | 100,000 b.b. | ++ | ++ | - | - | - | - | - | - | - | - |
| | 10,000 b.b. | + | + | + | + | + | + | + | + | + | + |
| | 1,000 b.b. | ++ | ++ | + | + | + | + | + | + | + | + |
| | 100 b.b. | + | + | + | + | + | + | + | + | + | + |
| | 1 mill. b.b. | + | + | + | + | + | + | + | + | + | + |
| | 100,000 b.b. | ++ | ++ | - | - | - | - | - | - | - | - |
| | 10,000 b.b. | + | + | + | + | + | + | + | + | + | + |
| | 1,000 b.b. | ++ | ++ | + | + | + | + | + | + | + | + |

Symbols: + = growth in liquid yolk medium.
 ++ = growth in McCoy's medium.

Table 4.
Isolating *Pasteurella tularensis* in liquid egg yolk cultures inoculated
with organ fragments from dead animals.

| a) White mouse No. 100 | | | | | | |
|--------------------------|-------------------|----------------------|--------|-------|-------|-------|
| Medium | Growing period | Inoculation material | | | | |
| | | Glands | Spleen | Liver | Lungs | Blood |
| Liquid egg yolk m. | 24 hrs | + | —* | + | + | + |
| | 4 days | + | + | + | + | + |
| Coagulated McCoy's m. | 24 hrs | — | — | — | — | — |
| | 4 days | + | + | — | — | — |

*The test tube with material from the spleen was cracked. It overgrew
with extraneous bacteria so that a *Pasteurella tularensis* culture
could not be isolated.

| b) Guinea pig No. 97 | | | | | | |
|--------------------------|--------|---|---|---|---|---|
| Liquid egg yolk m. | 24 hrs | + | + | + | + | + |
| | 4 days | + | + | + | + | + |
| Coagulated McCoy's m. | 24 hrs | — | — | — | — | — |
| | 4 days | — | + | — | — | — |

It was easier, therefore, to isolate *Pasteurella tularensis* from
liquid egg yolk cultures produced by organ fragments from animals dead of
tularemia than from similar cultures in McCoy's medium.

Bact. tularensis isolated in liquid egg yolk medium
seeded with organ fragments from sacrificed animals.

The experiments in question were conducted on white mice and guinea
pigs. The first group of animals consisted of 5 white mice, each of which
was subcutaneously inoculated with a dose of 100 million bacterial bodies.

In the second group, the mice were infected with considerably smaller doses (1,000 bacterial bodies) of the same Bact. tularensis strain. In this test, the superiority of the liquid egg yolk medium was brought out even more forcefully. For example, inoculation material from animals which had been sacrificed 6 to 24 hours after the infecting injection, produced *Pasteurella tularensis* cultures only in the liquid medium, but none in McCoy's medium. In the case of animals killed 2 days after the infecting injection, cultures were isolated in the liquid medium by inoculating it with material from the injection site and with blood, while McCoy's medium produced cultures only when it was inoculated with the blood. Additional data on these tests can be found in Table 5.

| | Time after infection | Infection site | | Glands | | Spleen | | Liver | | Lung | | Blood | |
|---------|----------------------|-----------------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|
| | | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. |
| | | Dead Sacrificed | 6 hrs | + | - | - | - | - | - | - | - | - | - |
| 24 hrs. | + | - | + | - | - | - | - | - | - | - | - | + | - |
| 48 hrs. | + | - | + | - | - | - | - | - | - | - | - | + | + |
| 96 hrs. | + | - | + | - | + | + | + | + | + | + | - | + | + |
| Dead | 168 hrs | + | - | + | + | + | + | + | + | + | - | - | - |

| Mice infected with glanders (inocul. dose 1,000 b.b.v.) | | | | | | | | | | | | | | |
|---|----------------------|----------------|-------|--------|-------|--------|-------|-------|-------|------|-------|-------|-------|--|
| | Time after infection | infection site | | Glands | | Spleen | | Liver | | Lung | | Blood | | |
| | | Liq. | McCoy | Liq. | McCoy | Liq. | McCoy | Liq. | McCoy | Liq. | McCoy | Liq. | McCoy | |
| | | med. | med. | med. | med. | med. | med. | med. | med. | med. | med. | med. | med. | |
| Dead Sacrificed | 6 hrs | + | - | - | - | - | - | - | - | - | - | - | - | |
| | 24 hrs. | + | - | + | - | - | - | - | - | - | - | + | - | |
| | 48 hrs. | + | - | + | - | - | - | - | - | - | - | + | + | |
| | 96 hrs. | + | - | + | - | + | + | + | + | + | - | + | + | |
| Dead | 168 hrs | + | - | + | + | + | + | + | + | + | - | - | - | |

Experiments in which guinea pigs were subcutaneously infected with individual doses of 500 million bacterial bodies fully confirmed the experimental results in white mice. Pure Bact. tularensis cultures were yielded by material from the injection site and glands of one animal killed 6 hours and another killed 24 hours after the infecting injection; material from a third (injection site, glands, liver, blood), killed 48 hours after the injection, produce Bact. tularensis cultures, but only on the liquid medium. On McCoy's medium there was absolutely no proliferation. On this medium, Bact. tularensis was successfully cultured only from one guinea pig which had been killed 5 days after the infection.

The corresponding results are presented in Table 6.

Table 6.
Tests on guinea pigs infected with individual subcutaneous doses of
500 million bacterial bodies

| Time after Injection infection | | site | | Glands | | Spleen | | Liver | | Lung | | Blood | |
|-----------------------------------|---------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|
| | | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. | Liq. med. | McCoy med. |
| Sacrificed | 6 hrs | + | - | + | - | - | - | - | - | - | - | - | - |
| | 24 hrs | + | - | + | - | - | - | - | - | - | - | - | - |
| | 48 hrs | + | - | + | - | - | - | + | - | - | - | + | - |
| | 120 hrs | + | + | + | + | + | - | + | - | - | - | + | + |
| | 288 hrs | + | - | + | - | + | - | + | - | - | - | - | - |

Symbols: + = first signs of growth.

- = no growth.

It should be mentioned that Bact. tularensis cultures were obtained (but only in the liquid egg yolk medium) from the organs of a guinea pig which was chloroformed on the 12th day, having survived the infection.

The 10% egg yolk medium is better than McCoy's medium for culturing Pasteurella tularensis from the organs of animals which have either died of tularemia or which are killed at various stages of the disease, because it requires smaller inoculation doses and because the Bact. tularensis grows in it faster. This statement is borne out by the results of experiments in which the minimum inoculation dose for test tube cultures was established.

This last series of experiments fully confirms the fact that in animals which have died of tularemia, or which are infected with tularemia, the bacteriological diagnosis can be more rapidly established by making use of the liquid egg yolk medium.

Conclusions.

1. The liquid egg yolk culture medium, having been investigated as a potential culture medium for Bact. tularensis, it was found that it is excellent for isolating this bacterium from animal sources.
2. The optimum egg yolk concentration of the physiological solution is 10%.
3. The liquid egg yolk medium requires bacterial inoculation doses which are 100 times lower than those required by McCoy's medium, and Bact. tularensis proliferates in it much more rapidly than in the latter.
4. Bact. tularensis survives in the liquid egg yolk medium for a long time (5 months), even under unfavorable storage conditions.
5. The liquid egg yolk medium is better than McCoy's coagulated egg yolk medium for isolating Bact. tularensis in biological test animals. The bacterium is cultured in it from dead or killed animal sources more rapidly and the culturing results are positive in a larger number of cases.
6. It can be hoped on the strength of the present experimental results that it will be possible to obtain Bact. tularensis cultures in the liquid medium directly, without animal passages and furthermore

that during epizootic periods and outbreaks of tularemia among people it will be possible to isolate it even from human sources.

7. The liquid egg yolk medium is more economical than McCoy's medium, its preparation requiring less egg yolk material.

8. The only drawback of the liquid egg yolk medium is that it is opaque so that bacterial growth cannot be macroscopically observed in it.

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